Measurement of Serum Glucose with Oxidase Immobilized onto Onion Membrane

Sanjay Kumar
Biochemistry Research Laboratory,
Department of Biochemistry & Genetics
M.D. University, Rohtak

C.S. Pundir
Biochemistry Research Laboratory,
Department of Biochemistry & Genetics
M.D. University, Rohtak

Abstract: *A reusable strip of glucose oxidase (GOD) from Aspergillus was prepared by immobilizing the enzyme onto onion membrane affixed on plastic strip with a conjugation yield of 2.0 mg/cm^2 and 63% retention of its initial activity. The immobilized enzyme showed maximum activity at pH 6.5, when incubated at 37°C for 15 min. A method for measurement of serm glucose was developed employing this strip. The minimum detection limit of the method was 5.0 mg/dl. Within batch and between batch CV for serum glucose were <5% and <6%. A good correlation(r=0.90) was found between serum glucose by commercial enzo kit method and the present method. No significant loss in the strip was observed after its 100 regular uses over a period of 40 days, when stored in reaction buffer at 4°C. This method has advantage over commercial enzymic colourimetric method that it provides reuse of glucose oxidase with ease.*

Keywords: *Glucose, glucose oxidase, immobilization, onion membrane, serum.*

1. Introduction

Measurement of glucose in serum is required for the diagnosis and medical management of diabetes mellitus, hyper and hypo activity of thyroid, pituitary and adrenal gland and myoxydema conditions interfering with glucose absorption. Enzymatic colorimetric method, employing glucose oxidase and peroxidase though is comparatively simpler, sensitive, specific and requires only a colorimeter [1], yet it is more expensive for routine use due to high cost of bulk quantity of enzyme. The immobilization of the enzyme onto insoluble support permits its reuse and thus reduces the cost of procedure for a large number of clinical samples. Glucose oxidase from different sources has been immobilized onto various supports such as glass [2], alkylamine glass beads [3], nylon tubes [4], polythene films [5], radio frequency plasma modified poly(ether) urethane urea [6], polypropylene glass beads surface by covalent [7], nafion membrane [8], graphite [9], polypyrrole/polytetrahydrofuran graft copolymer [10], electrode surface [11], egg membrane [12], and many more either alone or with peroxidase for determination of glucose [13]. All these supports used for immobilization of glucose oxidase suffer from one drawback or the other, such as high cost, limited availability and susceptibility to microbial attack or complicated procedure of their preparation. The present work was therefore carried out to immobilize GOD on a easily available cheaper support i.e., onion membrane affixed on a plastic strip by non-reactive fixative.

Materials and Methods

Glucose oxidase from Aspergillus niger, horseradish, peroxidase and dextrose (SRL Mumbai), Enzo kit for glucose (ERBA India), 4-aminophenazone and glutaraldehyde (SIGMA, USA) were used. Onion bulb and commercial fixative were purchased from local market.

Preparation of reusable strip for glucose oxidase : The rectangular strips of 15 × 1 cm size were cut from a plastic sheet (Thickness: 0.5mm). One end of each strip was made round with the help of scissor.

Affixation of onion membrane on plastic strip : The round end of the plastic strip was scratched with sandpaper on both sides up to a height of 2 cm. A thin layer of 0.1mm thickness of non reactive fixative was spread uniformly on this scratched strip. An onion membrane was picked up from onion bulb and affixed uniformly on this fixative layer. This strip was kept for 24 hr at room temperature for affixation of membrane.

Immobilization of glucose oxidase onto affixed onion membrane : Activation of affixed onion membrane was carried out as described [14], The end of plastic

strip containing affixed onion membrane was dipped into a test tube containing 5ml glutaraldehyde solution (2.5% in 0.1M sodium phosphate buffer pH 7.0). The onion membrane was allowed to get activated for about 2 hr at room temperature with occasional stirring. The excess of glutaraldehyde was washed with 0.1M sodium phosphate buffer (pH 7.0) 7 to 9 times until the pH of discard was 7.0. The end of the strip containing affixed onion membrane was dipped into 2.0ml glucose oxidase (1.0mg/dl) in 0.1M sodium phosphate buffer (pH7.0) in 15 ml test tube (size: ODxlength = 2.5 x 5.7cm). It was kept for 48 hr at 4°C with occasional shaking. The strip was taken off from enzyme solution, which was tested for enzyme activity. The strip was dipped into distilled water 5-6 times until no activity of enzyme was detected in the consequent washing. The enzyme bound to onion membrane was measured by determining the protein in the enzyme preparation before and after immobilization [15].

Assay of strip bound glucose oxidase : The plastic strip containing immobilized glucose oxidase onto affixed onion membrane was termed as Enzyme strip. The assay was carried out in a 15ml test tube wrapped with black paper. To 1.9ml 0.1M sodium phosphate buffer (pH7.0), the enzyme strip was inserted in such a manner that its end containing immobilized enzyme was dipped in the reaction buffer. After preincubation at 37°C for 5 min, the reaction was started by adding 0.1ml glucose solution (1mg/ml). After incubating the reaction mixture at 37°C for 5 min under gentle and continuous stirring in water bath shaker, the enzyme strip was taken off and 1.0ml color reagent consisting 18mg 4-aminophenazone, 36mg solid phenol, and 1.0mg horseradish peroxidase in 100ml 0.4M sodium phosphate buffer, pH-7.0 was added and kept at room temperature for 15min. The reaction mixture was transferred to a cuvette and A_{520} was read in Spectronic-20 (Million & Roy, USA) against control. The control was prepared in the same manner except that strip had only affixed onion membrane. Both the enzyme and control strips were washed in distilled water after the assay stored in 0.1M sodium phosphate buffer (pH-7.0) at 4°C.

Kinetic properties of strip bound GOD : Following kinetic properties of immobilized enzyme were studied- optimum pH, incubation temperature, time of incubation, effect of glucose concentration and calculation of Km and Vmax (from L-B plot).

Determination of serum glucose with GOD strip : Blood sample (1ml) from apparently healthy adults and persons suffering from diabetes mellitus of different age group and sex were collected using sterilized need and syringe and kept at room temperature for 1hr. Afte centrifuging at 2000rpm for 5min at room temperatur the supernatant (serum) was collected and diluted i reaction buffer in 1:1 ratio. The assay of serum glucos was carried out in the same manner as described fo assay of strip bound GOD under optimum assa conditions except that the glucose solution was replace by diluted serum. The glucose concentration in serur was extrapolated from standard curve between glucos concentration ranging from 20 to 12 mg/dl and A_{5} prepared under optimal assay condition.

Reuse and storage of enzyme strip : To reuse th enzyme strip, its end containing immobilized enzym was washed off with distilled water 5-6 times prior t its use in next assay. The enzyme strip was stored a 4°C in reaction buffer when not in use.

Results and Discussion

Commercially available GOD from Aspergillu niger was immobilized through glutaraldehyde couplin onto onion membrane affixed on a plastic strip by nor reactive fixative with 63% retention of initial activity c free enzyme and 2.0mg/cm^2 conjugation yield (Table 1 The enzyme was immobilized covalently onto onio membrane through glutaraldehyde between-NH_2 group on the surface of enzyme and affixed onion membran

Table 1: Immobilization of glucose oxidase from *Aspergillus niger* onto onion membrane affixed on a plastic strip

Enzyme added to membrane (mg proteins)	4.5
Enzyme coupled to membrane (mg proteins)	2.9
Enzyme unit added (nmol H_2O_2/mL)	720
% Enzyme coupled	2.0
% Retention of sp. Activity	63

Kinetic properties of immobilized GOD : Table summarizes the comparison of kinetic properties of fre and onion membrane bound GOD. Compared to fre enzyme, the membrane bound GOD showed an increas in optimum pH from 5.0 to 6.5. A similar increase in p of GOD (pH 5.0 to 6.5) has been reported for enzyn sensor based on GOD immobilized on Sigma glas supported aminopropy (15). An increase in Km valu for glucose from 5.5 to 11.0 mM and Vmax from 0.1 to 0.95 nmol H_2O_2/min were observed but no change time of incubation was observed after immobilize similar to that for enzyme bound to affixed eg membrane [12]. The change in kinetic properties

Table 2: Kinetic parametres of free and immobilized glucose oxidase from Aspergillus niger onto onion membrane affixed on a plastic strip

Parameter	Free	Immobilized
Optimum pH	5.0	6.5
Temperature for maximum activity	37°C	37°C
Time of incubation (min.)	20	20
Saturating conc. of glucose (mM)	9	20
Km for glucose (mM)	5.5	11.0
Vmax (μmol/min)	0.11	0.95

Data are mean of three replicates

immobilized enzyme might be due to change in the enzyme conformation, steric hindrance, micro-environmental effect and bulk and diffusional effects after immobilization [17].

Determination of serum glucose : An enzyme colourimetric method for discrete analysis of glucose in serum was developed which provides the reuse of enzyme (GOD) with ease. Further, the method provides an easily available cheaper support for immobilization of GOD and is free from possible interference of immobilized enzyme system by accumulation of product of colour-reaction due to its repeated use. Following parameters were studied to evaluate this method.

Linearity : A linear relationship was found between A_{520} versus glucose conc. ranging from 20mg/dl to 120mg/dl in reaction mixture, which is similar to that by co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (20-100 mg/dl) (15) but higher than that by enzymic sensor (5-20mg/dl) (17).

Detection Limit : The lower detection limit of the method was 5mg/dl, which is similar to that by co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (5mg/dl) (7) but higher than that by GOD and horse radish peroxidase immobilized individually onto alkylamine glass beads (3.6mg/dl) (3) and lower than that by direct fluorometric determination (8.5mg/dl) (7). The upper detection limit of the method is 120mg/dl. As the serum was diluted in 1 : 1 ratio before measurement of glucose, the method was able to measure glucose in serum up to 240mg/dl.

Analytical recovery : The mean analytical recovery of added solid glucose into serum (50mg/dl) was (82±0.70%) (mean ± SD; n=6) which is comparable to that of using co-immobilized glucose oxidase and peroxidase onto arylamine glass beads affixed on a plastic strip (90.3%) (16).

Precision : To check the reproducibility and reliability of the method, glucose content was measured in the serum sample six times in one run (within batch) and serum samples after one week of storage at-20°C (between batch). The within and between day coefficient of variation (CV) for serum glucose determination were <5.0 and <6.0% respectively, similar to that for egg membrane bound GOD (12) but lower to that by co-immobilized glucose (<5.6% for within batch and <10% for between batch) (16) indicating good reproducibility and reliability of the method.

Accuracy : The serum glucose in normal persons as measured by commercial enzo kit method and the present method showed a good correlation (r=0.90) indicating high accuracy of the method.

Determination of serum glucose : The glucose value in serum obtained from normal and diabetic individuals of various age groups and sex was measured by the present method which is ranged from 60 to 100 mg/dl with a mean of 75.4mg/dl in healthy (n=50) and 140 to 240mg/dl in diabetic persons. (n=50)

Reusability and storage stability of GOD strip : The immobilized enzyme strip did not show any considerable loss of its activity after its regular use over a period of 40 days, when stored in 0.1M reaction buffer at 4°C. During this period the enzyme strip was used for 100 times.

In conclusion, a reusable GOD strip was prepared for serum glucose determination. The strip has advantage over commercial enzymic method, as it provides the reuse of GOD with ease.

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